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Comparative gene expression of gonadotropins (FSH and LH) and peptide levels of gonadotropin-releasing hormones (GnRHs) in the pituitary of wild and cultured Senegalese sole (*Solea senegalensis*) broodstocks

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Abstract

The Senegalese sole (*Solea senegalensis*) is a valuable flatfish for aquaculture, but it presents important reproductive problems in captivity. Spawning is achieved by wild-caught breeders but cultured broodstock fail to spawn spontaneously and when they do eggs are unfertilized. To gain knowledge on the physiological basis underlying this reproductive dysfunction, this study aimed at analyzing comparative hormone levels between wild and cultured broodstock at the spawning season. The S. sole gonadotropin (GTH) subunits, FSHβ, LHβ and GPα, were cloned and qualitative (in situ hybridization) and quantitative (real-time PCR) assays developed, to analyse pituitary GTH gene expression. In females, FSHβ and GPα mRNA levels were higher in wild than in cultured broodstock, whereas in males all three subunits were highest in cultured. By ELISA, three GnRH forms were detected in the

pituitary, displaying a relative abundance of cGnRH-II> sbGnRH > sGnRH. All GnRHs were slightly more abundant in wild than cultured females, whereas no differences were observed in males. Plasma levels of vitellogenin and sex steroids were also analyzed. Results showed endocrine differences between wild and cultured broodstock at the spawning period, but they were probably not critical to determine an endocrine failure of the reproductive axis in cultured breeders.

Key words: FSH; gonadotropin; GnRH; LH; reproductive dysfunction; Senegalese sole; *Solea senegalensis*; spawning season.

Introduction

The brain-pituitary-gonad (BPG) axis regulates the reproductive function in vertebrates, including fish. External and internal signals are primarily integrated in the brain by the hypothalamic neuroendocrine neurons producing the gonadotropin-releasing hormones (GnRHs). The GnRHs stimulate the synthesis and secretion of gonadotropins (GTHs) in the pituitary, which are critical modulators of gametogenesis and gonadal maturation through the mediation of gonadal steroidogenesis and growth factors (Schulz and Miura 2002; Yaron et al., 2003).

Three GnRH forms, seabream GnRH (sbGnRH), salmon GnRH (sGnRH) and chicken-II GnRH (cGnRH-II) have been found to coexist in the brain of several teleosts, including flatfishes (Lethimonier et al., 2004; Pham et al., 2006). The sbGnRH is found to be the predominant form in the pituitary and considered the most relevant GnRH in the control of GTH synthesis and secretion (Powell et al., 1994; Gothilf et al., 1997), whereas the gonadotropic role of sGnRH and cGnRH-II may vary among species (Holland et al., 1998, 2001; Rodríguez et al., 2000; Andersson et al., 2001).

The GTHs, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), are heterodimers which share a common α subunit (GPα) and posses a hormone specific β subunit (FSHβ and LHβ) (Pierce and Parson, 1981; Yaron et al., 2003). In salmonids, FSH predominates during vitellogenesis and spermatogenesis, while secretion of LH peaks at final gonadal maturation, ovulation and spermiation (Gómez et al., 1999; Swanson et al., 2003). In non-salmonid species, information on GTH releasing profiles is mostly restricted to LH because of the limited availability of FSH immunoassays. However, some information on the bioactivity of both GTHs has been obtained through molecular approaches, by analyzing gene expression levels of their subunits. Among non-salmonid species, single spawners have shown a temporal

gene expression of FSHβ and LHβ similar to those of salmonids, with a clear prevalence of LHβ gene expression at final stages of gonadal maturation (Yoshiura et al., 1999; Kim et al., 2005). On the other hand, multiple spawners seem to express a progressive and simultaneous increase of both FSHβ and LHβ gene expression during gonadal maturation (Elizur et al., 1996; Sohn et al., 1999; Jackson et al., 1999; Mateos et al., 2003; Kajimura et al., 2001; Weltzien et al., 2003; Meiri et al., 2004).

Optimal rates of synthesis and secretion of both GnRHs and GTHs are critical for successful gonadal maturation and spawning (Peter and Yu, 1997; Zohar and Mylonas, 2001). In aquaculture, fish often exhibit some degree of reproductive dysfunction, most commonly, absence or reduced egg quality and quantity in females and diminished milt production in males (Mylonas and Zohar, 2001). Failure to spawn in captivity has been associated to inhibited LH release from the pituitary (Zohar, 1988; Mylonas et al., 1997, 1998). Further studies have suggested a negative effect of confinement directly onto sbGnRH mRNA and pituitary peptide levels (Zohar and Mylonas, 2001). Therefore, the brain-pituitary endocrine system seems to be impaired under culture conditions, affecting adversely the normal functioning of the endogenous GnRH and GTH system and thus the reproductive process.

The Senegalese sole (*Solea senegalensis*), S. sole, is a highly valuable fish which has become a priority species for European and Mediterranean aquaculture diversification (Imsland et al., 2003; Cañavate, 2005). However, the current production of this flatfish is largely based on wild-caught breeders, which display spontaneous fertilized spawning under rearing conditions (Dinis et al., 1999; Anguis and Cañavate, 2005). Contrarily, cultured S. sole breeders (hatched and raised in captivity) fail to spawn spontaneously and when they do, spawning is of low quantity and eggs are generally found unfertilized (García-López et al., 2007; Guzmán et al., 2008). The causes of these generational differences, even when wild and cultured broodstocks are reared under similar culture conditions for several years, are unknown.

A number of recent studies have described several aspects of S. sole reproduction, including spawning performance and its correlation with annual profiles of plasma VTG and sex steroids in both wild (Anguis and Cañavate, 2005; García-López et al., 2006a) and cultured broodstocks (García-Lopez et al., 2006b, 2007; Guzmán et al., 2008). However, there is no information on the levels of GnRHs and GTHs in S. sole, neither endocrine studies aimed directly to compare wild and cultured breeders. Recently, the molecular cloning of S. sole GTHs has been presented (Guzmán et al., 2007; Cerdá et al., 2008), and information on GTH gene expression in cultured males given (Cerdá et al., 2008).

The purpose of the present study was to analyze pituitary GTH gene expression and pituitary GnRH content on paralleled wild and cultured S. sole broodstock at the spawning time, in an attempt to detect an endocrine failure which might be related to the reproductive dysfunction of cultured breeders. For this, we first cloned and developed real-time quantitative PCRs (qPCRs) for the S. sole FSHβ, LHβ and GPα subunits. Analysis of GTH subunit mRNA levels and pituitary GnRH content (by ELISA) were performed in both females and males, and correlated with VTG and sex steroid plasma levels, and gonadal histology. The cloning of S. sole GTHs also allowed studying the distribution of GTH subunit gene expression cells in the pituitary of males and females, by in situ hybridization.

Materials and Methods

Animal housing and sampling

The Senegalese sole (*Solea senegalensis*), S. sole, breeders used in this study were obtained from two broodstocks (wild and cultured) reared at the facilities of the private fish farm "Stolt Sea Farm s.a." (A Coruña, Spain, 42º N 8º W). Wild broodstock consisted of fish that were captured from the wild by fishermen as juveniles, in 2003, transported immediately to the abovementioned fish farm and further grown and reared at the holding facilities. Cultured broodstock consisted of female (5 years-old) and male (6 years-old) S. sole hatched and rose in captivity from spawns obtained from wild-caught breeders at the same fish farm. Both wild and cultured broodstocks were always kept at the same facilities and under similar culture conditions, maintaining both broodstocks in paralleled but separated tanks. Tanks were supplied with an open flow-through sea water system (salinity ~36‰, 3 renewals per day) and exposed to natural cycles of photoperiod and temperature. Tank densities were maintained at around 2 kg m^2 and sex ratios at around 1:1. Fish were fed daily *ad libitum* with dry pellets (ProAqua).

All tanks were equipped with egg collectors. Spawning was checked daily (twice) and quantity and quality of eggs recorded. The spawning period of wild broodstock took place from March to September, whereas the cultured broodstock produced some spontaneous but unfertilized spawning from April to June. Sampling was performed on the 28th of May 2007, considered as the full spawning period.

Female (n=4) and male (n=4) breeders were sampled from wild and cultured broodstock, identified through passive integrated transponder tags (pit-tags, AVID). For sampling, fish were deeply anaesthetised by immersion in 2-phenoxyethanol (3 ml l^{-1}) and body weight and length recorded. Blood (1 ml) was collected from the caudal vasculature

with heparinised syringes and placed on ice-cold heparinised tubes containing aprotinin (1 mM). Plasma was obtained by centrifugation (3000 *g*, 15 min, 4º C) and stored at -20º C, for further analysis. Fish were sacrificed by decapitation for dissection of tissues. Pituitary glands were collected, immediately frozen in liquid nitrogen and stored at -80ºC until RNA extraction. Gonads were removed and weighted for calculation of the gonadosomatic index (GSI = gonad weight x100 /body weight). For histology, a portion of the gonad was taken from the middle part and placed in fixative (4% formaldehyde, 1% gluteraldehyde). The gonad was further embedded in glycol methacrylate resin (Technovit 7100, Heraeus Kulzer, Germany), cut into 3 μm sections and stained with methylene blue/ azure II/ basic fuchsin (Bennet et al., 1976).

All procedures were carried out according to national and institutional regulations (Consejo Superior de Investigaciones Científicas, Institute of Aquaculture of Torre la Sal Review Board) and the current European Union legislation on handling experimental animals.

Molecular cloning of S. sole GTH subunits

For cloning, four pituitaries were collected from mature male (n=2) and female (n=2) S. sole. Fish were sacrificed with phenoxyethanol overdose and pituitary glands removed and immediately frozen in liquid nitrogen. Total RNA was extracted from the pooled pituitaries using RNeasy Mini kit (Quiagen). Reverse transcription was performed on 600 ng of total RNA with Powerscript Reverse Transcriptase (Clontech, Palo Alto, CA) according to the manufacturer's protocol for 5' and 3' first strand synthesis.

Complete cDNA sequences for FSHβ, LHβ and GPα subunits were obtained by two rounds of RACE-PCR, using the SMART^{TM}-RACE-PCR protocol (Clontech). Partial cDNA sequences were first isolated using primers FSH-FW, LH-RV and GP-RV (table 1). Thermal cycling parameters for FSHβ 3' and LHβ 5'-end amplification were similar to those described for the amplification of FSHβ and LHβ in three-spined stickleback (*Gasterosteus aculeatus*) (Hellquist et al., 2004). The 5'RACE-PCR for GPα amplification consisted of 5 cycles of 94 ºC for 30 s, 64 ºC for 30 s and 72 ºC for 45 s; 5 cycles of 94 ºC for 30 s, 62 ºC for 30 s and 72 ºC for 45 s; 35 cycles of 94 ºC for 30 s, 60 ºC for 30 s and 72 ºC for 45 s. The PCR products were cloned into pGEM-T Easy plasmid vectors (Promega, Madison, WI). Plasmid DNA obtained from transformed colonies (JM109 Competent Cells, High Efficiency, Promega) was purified using Nucleo Spin Plasmid QuickPure (Macherey-Nagel), and sequenced on an ABI Prism 310 Genetic Analyzer (Applied Biosystem). Partial cDNA sequences were identified using the BLAST program (GenBank, NCBI). Based on the obtained partial cDNA sequences, gene specific primers were designed (ssFSHr, ssLHf and ssGPf, see Table 1) and a

second round of RACE-PCRs performed. The 5'-RACE-PCR for FSHβ consisted of 5 cycles of 94 ºC for 30 s, 66 ºC for 30 s and 72 ºC for 2 min; 5 cycles of 94 ºC for 30 s, 64 ºC for 30 s and 72 ºC for 2 min; 35 cycles of 94 ºC for 30 s, 62 ºC for 30 s and 72 ºC for 2 min. The 3'-RACE PCRs for LHβ and GPα were, for both, 5 cycles of 94 ºC for 30 s, 68 ºC for 30 s and 72 ºC for 2 min; 5 cycles of 94 ºC for 30 s, 66 ºC for 30 s and 72 ºC for 2 min; 35 cycles of 94 ºC for 30 s, 64 ºC for 30 s and 72 ºC for 2 min.

The partial 5'- and 3'-end cDNA sequences from each subunit were assembled and the complete nucleotide sequence identified using BLAST. The amino acid sequences were deduced by ExPASY (www.expasy.org/tools/dna.htmland) and the signal peptide and putative cleavage sites predicted using the SignalP 3.0 server program (www.cbs.dtu.dk/services/SignalP). Potential N-glycosylation sites were identified by searching for the motif Asn-Xaa-Ser/Thr using the program Gene Runner version 3.02. Alignment of sequences and analysis of identities was performed using ClustalW multiple sequences alignment program.

Real-time quantitative PCR (qPCR)

Plasmids containing the FSHβ, LHβ and GPα insert were linearized and used as templates for gene-specific RNA standard synthesis. Transcription was performed in a 30 µl volume reaction, containing: 1 µg plasmid DNA template, 1mM rNTP, 1mM DTT, 1.5 U μl^{-1} RNA polymerase and 1.3 U μl^{-1} RNase OUT (Invitrogen). Residual DNA was destroyed with DNase (RNase-free, 2 U/ 50 µl, for 30 min at 37 ºC), and RNA purified via Chroma Spin-200 (BioScience). Total RNA isolated from a pool of S. sole pituitaries, using Tri-Reagent (Sigma) and treated with DNase, served as the standard for 18s RNA. The amount of each RNA standard was determined using RiboGreen RNA quantification kit (Molecular Probes, Eugene, OR).

Validation assays for each target (FSHβ, LHβ and GPα) and the reference gene (18s) were conducted using serially diluted RNA standards as template. RNA standards were first reverse transcribed to cDNA with random hexamers and MMLV reverse transcriptase (Promega) and used as template in qPCR, using the SYBR Green PCR core reagent (Applied Biosystem) containing 200 nM gene-specific primers (Table 2). Amplification reactions were carried out at 50 ºC for 2 min, 95 ºC for 10 min, and 40 cycles of 95 ºC for 15 s and 60 ºC for 60 s, using an ABI Prism 7700 Sequence Detection System. After the amplification phase, a dissociation step was carried out at 95 ºC for 15 s, 60 ºC for 15 s, and 95 ºC for 15 s. Reaction efficiencies were calculated based on the second derivative maximum ($E=10^{-(1/slope)}-1$).

For sample analysis, total RNA was isolated from each individual S. sole pituitary using the Tri-Reagent, treated with DNase and quantified (NanoDrop ND-1000 Spectrophotometer). RNA standards and RNA from each sample were reverse-transcribed simultaneously and analyzed by qPCR as abovementioned. After the qPCR reaction, copy number for unknown samples was determined by comparing C_T (threshold cycles) values to the specific standard (run in every plate) and normalized to the amount of 18s RNA in each sample.

In situ hybridization

For *in situ hybridization* (ISH), pituitary glands were collected from female and male wild breeders and kept in freshly prepared sterile paraformaldehyde at 4ºC overnight. Afterwards, samples were dehydrated with increased methanol concentrations, washed 3x10 min with 0.1 M sodium phosphate buffer, pH 7.2 and defatted with xylene before embedding in paraffin. Longitudinal sections, 6-8 µm thick, were mounted on slides coated with 3-triethoxysilyl propylamine (Merk, Darmstadt, Germany). The FSHβ (spanning from 109 to 555 bp), LHβ (spanning from 166 to 578 bp) and GPα (spanning from 258 to 756 bp) subunit cDNA fragments were subcloned into pGEM-T easy (Promega). Linearized cDNAs were used to prepare digoxigenin 11-UTP-labelled single-strand RNA probes with the DIG RNA labelling kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Treatment of pituitary histological sections and hybridization conditions were similar to those previously described; the controls included hybridization with sense probes, RNAse treatment before hybridization and absence of the antisense RNA probe/anti-digoxigenin antibody in the incubation reaction (Ortiz-Delgado et al., 2005).

GnRH ELISAs

The pituitary peptide levels of sbGnRH, sGnRH and cGnRH-II were measured in the same pituitaries used for GTH mRNA analysis; in the protein fraction obtained after the RNA extraction. Proteins were precipitated with iso-propanol (1:2, v/v) and pellets dried and reconstituted in 200 µl PBST buffer (10 mM phosphate buffer, pH 7.2, containing 0.9% NaCl and 0.05% Tween 20). Acetic acid 4N was added to each sample (1:1, v/v), incubated for 10 min at 80 ºC and centrifuged (13,000 g, 30 min, 4ºC). The pellets were re-extracted twice with acetic acid 4N. Supernatants were pooled, vacuum dried and reconstituted with potassium phosphate buffer (0.1 M, pH 7.4).

Levels of sbGnRH, sGnRH and cGnRH-II were measured in each reconstituted sample using specific ELISAs for each GnRH form. The ELISA protocol was that originally developed for gilthead seabream (*Sparus aurata*) (Holland et at, 1998), and further used for European sea bass (*Dicentrarchus labrax*) (Rodríguez et al., 2000), turbot (*Scolphtalmus maximus*) (Andersson et al., 2001), stripped bass (*Morone saxatilis*) (Holland et al., 2001) and zebrafish (*Danio rerio*) (Steven et al., 2003). The sensitivities of the ELISAs, determined at 80% of binding, were 6 pg $well^{-1}$ for sbGnRH, 7 pg $well^{-1}$ for cGnRH-II and 2 pg $well^{-1}$ for sGnRH. Cross-reactivities, calculated at 50% of binding, were below 0.6% except for the sGnRH assay which displayed 3.7% cross-reactivity with cGnRH-II.

S. sole VTG and sex steroid ELISAs

Plasma levels of VTG were measured by homologous ELISA, using purified S. sole VTG as standard and specific antibodies (Guzmán et al., 2008). The sensitivity, checked by means of lowest detection limit (Bo-2SD, maximum binding minus twice the standard deviation), was 3.6 ng ml^{-1}. Intra- and inter-assay coefficient of variations were 6.7% (n=12) and 9.8% (n=29), respectively.

For steroid analysis, plasma samples were first extracted with alcohol. Ice cold methanol was added to the plasma (6:1, v/v), shaken and centrifuged (3,000 g, 15 min, 4 ºC). The pellet was re-extracted twice with 200 µl of methanol. Supernatants were pooled, dried and reconstituted in 0.1 potassium buffer (pH 7.4). Levels of estradiol (E2), testosterone (T) and 11-ketotestosterone (11-KT) were quantified by ELISA using a protocol previously validated for S. sole plasma samples (Guzmán et al., 2008). The sensitivities of the ELISAs, calculated as the lowest detection limit (Bo-2SD), were 5.2 pg ml^{-1}, 8.8 pg ml^{-1}, and 0.4 pg ml^{-1} for the E2, T and 11-KT ELISA, respectively. The intra-(n=4) and inter-assay (n=8) coefficients of variation, at 50% of binding, were 5.8% and 6.3% for E2, 6.1% and 11.3% for T, and 10.7% and 9.1% for the 11-KT ELISA.

Statistical analysis

Data are expressed as mean ± standard error of the mean (S.E.M.). Differences wild versus cultured, and females versus males were examined using a t-test with a significance level of $p<0.05$. Differences among GTH subunit mRNAs and GnRH peptide levels were examined using a one-way ANOVA, followed by Student-Newman-Keuls (SNK) multiple comparison procedure, with a significance level of $p <0.05$. Normality and homogeneity of variance were tested by the Kolmogorov–Smirnov and Bartlett methods, respectively.

Results

Molecular cloning of S. sole gonadotropin subunits, development of molecular assays and localization of gonadotropin expressing cells by in situ hybridization (ISH)

Full-length cDNAs encoding the gonadotropin FSHβ, LHβ and Gpα subunits were isolated by 5' and 3' RACE-PCR, on reverse transcribed pituitary RNA (fig. 1). The full length FSHβ cDNA was 548 base pair (bp) long, containing a 363 bp long coding sequence flanked by a 5' untranslated region (UTR) of 100 bp, and a 3'UTR of 85 bp; it showed a consensus polyadenylation site (AATAAA) 18 bp upstream the poly(A) tail. The mature peptide consisted of 99 amino acids (aa), preceded by a 21 aa long signal sequence (fig. 1A). The complete LHβ cDNA was 571 bp long, and consists of a 21 bp 5'UTR, a coding sequence of 447 bp, and a 103 bp long 3'UTR; it showed two consensus polyadenylation sites located at 89 and 14 bp upstream of the poly(A) tail (fig. 1B). The mature LHβ peptide consisted of 124 aa preceded by a 24 aa long signal sequence. The 719 bp long GPα cDNA consists of a 32 bp long 5'UTR, and a 288 bp 3'UTR with a consensus polyadenylation signal 17 bp upstream the poly(A) tail. The 399 bp coding region encoded a mature peptide of 94 aa, which was preceded by a 38 aa long signal peptide (fig. 1C).

Comparison of the nucleic acid sequence in the coding region of the LHβ and FSHβ showed a similarity of 39%, whereas the deduced aa sequences were 22% identical between both subunits. The GPα subunit shared 35% and 32% similarity at the nucleic acid level and 7% and 9% at the amino acid level with LHβ and FSHβ, respectively.

The full cDNA sequences allowed development of qualitative (ISH) and quantitative (qPCR) molecular assays for detection of GTH subunit gene expression in the S. sole pituitary. Using specific probes, GTH subunit mRNA expressing cells was localized on pituitary parasaggital sections from male and female S. sole, by ISH. The GPα gene transcripts were detected in small basophilic cell populations located both in the border and in the medial region of the proximal pars distalis (PPD), and in the border of the pars intermedia (PI), as rounded and polygonal shape cell populations (fig. 2, A to E). Interestingly, FHSβ and LHβ expression was restricted to the border of both the PPD and the PI (fig. 2, G, I and J). No mRNA expression of GTH transcripts was detected in the rostral pars distalis (RPD) of the pituitary (fig. 2, A and B) from both female and male specimens. Sense riboprobes showed negative results (fig. 2, F, H and K). No differences in the pituitary distribution of FSHβ, LHβ or GPα gene transcripts were observed between males and females.

Using gene specific primers, qPCRs were developed for all three GTH subunits to quantify pituitary mRNA levels. In the set-up process special emphasis was dedicated to the accuracy of the assays, which was assured by using RNA from each target as standard, performing reverse transcription of both standard and samples simultaneously. Amplification efficiencies (%) for the qPCRs, obtained from validation assays using serially diluted RNA, were 102.3 ± 2.1, 102.5 ± 2.0 and 96.8 ± 3.5 for FSHβ, LHβ and GPα, respectively. A single peak was obtained for each gene during the melting curve analysis, demonstrating the specificity of the primers (data not shown).

Pituitary FSHβ, LHβ and GPα gene expression

Analysis of pituitary mRNA levels of the three GTH subunits in wild versus cultured S. sole broodstock is shown in fig. 3. Wild females showed higher FSHβ and GPα mRNA levels than cultured females ($p<0.05$); LHβ mRNA levels were similar. In males, both FSHβ and LHβ mRNA levels were 2-fold higher in cultured than in wild breeders, but these differences were not statistically significant.

Both FSHβ and LHβ subunits displayed similar mRNA levels in the pituitary of S. sole during the spawning time. This trend was observed in both genders and generations. In general, GPα subunit showed higher mRNA levels than GTHβ subunits, although these differences were only statistically significant ($p>0.05$) in wild females and cultured males.

No differences in FSHβ or LHβ mRNA levels were observed between females and males from the same generation.

Pituitary levels of GnRHs

By using specific ELISAs, all three GnRH forms, sbGnRH, sGnRH and cGnRH-II, were detected in the pituitary of S. sole at the spawning time (fig. 4). The relative abundance of the GnRH forms was: cGnRH-II > sbGnRH > sGnRH. However, statistical differences among the GnRH forms were only found in cultured females and wild males, which displayed a higher pituitary content of cGnRH-II ($p<0.05$).

Pituitary peptide levels of the three GnRHs were slightly higher, but not statistically significant, in wild than in cultured females. In males, no differences were observed in cGnRH-II or sbGnRH pituitary content between wild and cultured breeders. Cultured males had undetectable pituitary levels of sGnRH. The sGnRH was higher in females than in males, for both wild and cultured broodstock ($p<0.05$).

Gonadal histology and plasma levels of sex steroids and VTG

The histology of ovaries and testis collected from wild and cultured breeders at the time of spawning is shown in figures 5 and 6. The ovarian histology showed that both wild and cultured females were at a similar developmental stage, containing oocytes at advanced stages of maturation. According to the multiple-batch group synchronous type of ovarian development, other clutches of oocytes were also found at different stages of vitellogenesis (fig. 5, B and C). However, cultured females displayed a reduced proportion of mature oocytes in favour of those in vitellogenesis (fig. 5, G and H), whereas wild females showed a notorious abundance of maturing oocytes and, in some cases, postovulatory follicles (fig. 5, A to F) indicating recent spawning events.

Similarly, males showed fully mature gonads, as expected for the sampling time (fig. 6, A to D). All wild and cultured males displayed ripe accumulated spermatozoids in the medullar efferent ducts (fig. 6, A and B), but wild males showed increased lumen size and spermatozoid accumulation (fig. 6, A and B) than cultured (fig. 6, C and D). The GSI (fig. 7) was higher in wild than in cultured females ($p<0.05$); no differences where found in males from both groups.

Plasma levels of sex steroids and VTG are shown in figure 8. Wild and cultured females showed similar E2 and T plasma levels, but VTG levels were 3-fold higher in wild than in cultured females ($p<0.05$). In males, both sex steroids (T and 11-KT) were 2-fold higher in cultured than in wild breeders ($p<0.05$).

Discussion

This study described the molecular cloning of S. sole GTH subunits and development of quantitative real-time PCRs for analysis of GTH gene expression in the pituitary. Using the developed qPCRs, GTH mRNA levels were analyzed in females and males, comparing wild and cultured broodstocks at the spawning time. Also, inter-generational differences were examined by analysis of pituitary content of GnRHs (sbGnRH, sGnRH and cGnRH-II), plasma levels of VTG and sex steroids and gonadal histology and showed that there were significant endocrine and histological differences between wild and cultured broodstock during the spawning season.

The full cDNA sequences of the three GTH subunits were obtained by two rounds of RACE-PCR, from S. sole pituitary cDNA. Although previously presented in a Conference (Guzmán et al., 2007) and deposited in the GeneBank, this study showed the complete nucleotide sequences of S. sole FSHβ, LHβ and GPα subunits and adds new information to

373 the recently published deduced amino acid sequences of S. sole GTH subunits (Cerdá et al.,
374 2008). In the study by Cerdá et al., a different cloning strategy was used, using cDNA
375 libraries constructed from different larval stages and adult tissues of S. sole, but provided
376 strictly similar GTH amino acid sequences than those of the present study. The cloning of the
377 GTH subunits allowed development of specific qPCRs and thus, analysis of GTH gene
378 expression in the pituitary. At the spawning season, female S. sole breeders showed similar
379 transcript levels of FSHβ and LHβ in the pituitary, independently of the origin (wild or
380 cultured). These data are in agreement with previous studies performed in other multiple
381 spawning fish, which showed paralleled FSHβ and LHβ subunit gene expression during the
382 spawning season, such as goldfish (*Carassius auratus*) (Sohn et al., 1999), blue gourami
383 (*Trichogaster trichopterus*) (Jackson et al., 1999), gilthead seabream (Elizur et al., 1996,
384 Meiri et al., 2004) and the pleuronectiform Japanese flounder (*Paralichthys olivaceus*)
385 (Kajimura et al., 2001). In the present study, histological analysis of the gonads showed that
386 the ovary of S. sole contains clutches of oocytes at several stages of development throughout
387 the spawning season, which might require the action of both FSH and LH for continuous
388 recruitment, maturation and ovulation processes. This situation differs from single spawning
389 fish species, where LHβ gene expression highly predominates over that of FSHβ at the period
390 of gonadal maturation, ovulation and spawning, due to the predominant role of LH at final
391 stages of gonad maturation in these species (Gómez et al., 1999; Dickey and Swanson, 2000).
392 When comparing wild and cultured broodstocks, FSHβ mRNA levels were higher in wild
393 than in cultured females. Higher FSH gene expression may suggest higher FSH secretion
394 from the pituitary and thus, higher levels of the FSH protein in the bloodstream (Naito et al.,
395 1991; Dickey and Swanson, 2000; Gen et al., 2000), although this could not be confirmed in
396 S. sole because of the unavailability of FSH immunoassays. In our study, the higher pituitary
397 FSHβ transcript levels in wild females were correlated with higher plasma levels of VTG and
398 higher GSI. Considering the role of FSH in the regulation of VTG synthesis and VTG oocyte
399 uptake (Tyler et al., 1991; Jalabert, 2005), our results could indicate more active
400 vitellogenesis in wild than in cultured females. In the wild broodstock, the spawning period
401 lasted from March to September, as previously reported by other authors (Dinis et al., 1999;
402 Anguis and Cañavate, 2005). The detection of active vitellogenesis in wild females at the end
403 of May (sampling time 28th May) might resemble the oocyte recruitment required for multiple
404 spawns detected throughout the next few months. Contrarily, the cultured broodstock showed
405 a reduced duration of the spawning period, from April to early June, similarly to other reports
406 of cultured broodstock spawning (Guzmán et al., 2008). Therefore, a low vitellogenesis rate

and FSH synthesis at the end of May would be expected in this cultured broodstock, since scarce oocyte clutches should be preparing for further ovulations.

Contrarily to FSHβ, the LHβ transcript levels were similar in wild and cultured females. This result was somehow unexpected, due to the marked differences in spawning performance between both broodstocks. It is well known the predominant role of LH in the regulation of final gonad maturation, ovulation and spawning in fish (Nagahama, 1994; Peter and Yu 1997). Nevertheless, it would be possible that wild and cultured S. sole breeders presented differences in LH secretion, but this could not be detected in the present study because of the unavailability of a specific LH immunoassay. Previous works have described a lack of correlation between pituitary LHβ mRNA levels and circulating LH protein, indicating that synthesis and release of LH are differently regulated (Sohn et al., 1998, 1999; Swanson et al., 2003). Therefore, it would be possible that in our study, similar pituitary LHβ mRNA levels in wild and cultured females could occur together with marked differences in circulating LH plasma levels. Previous studies in wild and cultured stripped bass showed that pituitary LHβ mRNA levels and LH protein content were similar in wild and cultured females, but only wild females showed a plasma LH surge before spawning, whereas circulating LH remained low and unchanged in cultured females (Mylonas et al., 1997, 1998; Zohar and Mylonas, 2001). Development of an LH immunoassay will be necessary to study LH release deficiencies in cultured S. sole generations.

In males, the mRNA levels of FSHβ and LHβ at the spermiation period were similar. An active transcription of both GTHβ subunits during spermiation has been described in other multiple-spawning species, such as Japanese flounder (Kajimura et al., 2001), Atlantic halibut (*Hippoglossus hippoglossus*) (Weltzien et al., 2003), European sea bass (Mateos et al., 2003) and pejerrey (*Odontesthes bonariensis*) (Miranda et al., 2007). GTH gene expression levels have been recently reported in cultured male S. sole and showed also a similar expression of FSHβ and LHβ during spermiation (Cerdá et al., 2008). On the other hand, in salmonids it is known that pituitary FSHβ gene expression is clearly predominant during the early stages of gonad development declining during the period of spermiation, when LH synthesis and release are maximum (Gómez et al., 1999). The S. sole, as a multiple spawning species, the simultaneous expression of FSH and LH during the spermiation period should be necessary for the continuous stimulation of testicular maturation and spermiation throughout the spawning season.

The comparative analysis of pituitary GTH mRNAs in wild and cultured males showed that levels of all three subunits were slightly higher (2-fold; not statistically) in cultured than

441 in wild males. Also, this was correlated with increased plasma concentrations of both
442 androgens (T and 11-KT), in cultured as compared to wild breeders. In fish, it is well
443 established the role of FSH and LH as the primary regulators of spermatogenesis, mainly
444 through stimulation of gonadal biosynthesis of sex steroids (Schulz and Miura, 2002). Among
445 androgens, 11-KT stimulates Sertoli cell function that in turn promotes spermatogonial
446 proliferation and further steps along the process of spermatogenesis (Miura and Miura, 2003).
447 Therefore, the differences observed in our study could indicate that, at the spawning time,
448 cultured male breeders presented a less developed testicular stage than wild breeders. This
449 would agree with histological data that showed in the testis of cultured males a higher
450 presence of cells at early spermatogenesis (spermatogonia and spermatocytes) and lower
451 accumulation of spermatozoids.

452 Independently of the broodstock origin, the mRNA levels of both FSHβ and LHβ subunits
453 were similar in both male and female S. sole breeders. Some studies have reported sexual
454 dimorphism in FSHβ and LHβ gene expression throughout the fish reproductive cycle. In
455 gilthead seabream and red seabream (*Pagrus major*), at the spawning season, FSHβ mRNA
456 levels were higher in males than in females, whereas those of LHβ were similar in both
457 genders (Elizur et al., 1996; Gen et al., 2000). On the other hand, in goldfish, FSHβ mRNA
458 levels were higher in females than males at the spawning period, whereas similar LHβ mRNA
459 levels were found between sexes (Sohn et al., 1999). In three-spined stickleback, both GTHβ
460 subunits were higher in males than in females (Hellquist et al., 2006). Since our study was
461 limited to the spawning season, the lack of sexual dimorphism in GTHβ subunit gene
462 expression in S. sole should be confirmed in further studies.

463 Analysis of the distribution of gonadotropin expressing cells by in situ hybridization
464 showed no relevant differences between male and female S. sole. The GPα gene transcripts
465 were detected in small basophilic cell populations, located in the border and in the medial
466 region of the *proximal pars distalis* (PPD) and in the border of the *pars intermedia* (PI), as
467 rounded and polygonal shape cell populations. Interestingly, FHSβ and LHβ expression was
468 restricted to the border of both the PPD and the PI. According to Rendón et al. (1997),
469 gonadotropic cells in this species are basophilic cells, positive to PAS, Alcian Blue and lectin
470 reactions, containing glycoprotein rich in mannose and/or glucose and N-acetyl-glucosamine
471 and/or sialic acids sugar residues. These cells synthesize glycoprotein hormones located in
472 PPD and forming the external border of the PI.

473 The present study reported for the first time the coexistence of the three GnRH forms,
474 sbGnRH, sGnRH and cGnRH-II, in the pituitary of S. sole. A previous report described the

475 immunohistochemical distribution of GnRH in the brain of adult S. sole and found abundant
476 sGnRH and cGnRH-II immunoreactive fibres in the PPD of the adenohypophysis (Rodríguez-
477 Gómez et al., 1999), where gonadotropic cells were previously immuno-detected (Rendón et al.,
478 1997). The coexistence of sbGnRH, sGnRH and cGnRH-II in the brain/pituitary of fish has been
479 demonstrated in several fish species (Lethimonier et al., 2004), including the pleuronectiformes
480 turbot (Andersson et al., 2001), Barfin flounder (*Verasper moseri*) (Amano et al., 2004) and
481 Japanese flounder (Pham et al., 2006).
482 The peptide levels of each of the three GnRHs in the pituitary of S. sole were similar,
483 although their relative abundance could be rated as cGnRH-II > sbGnRH > sGnRH. This
484 situation differs from previous studies in perciformes (Senthilkumaran et al., 1999; Holland et
485 al., 2001; Rodríguez et al., 2001) and other pleuronectiformes. All three sbGnRH, sGnRH and
486 cGnRH-II forms were detected in the pituitary of barfin flounder, although peptide levels of
487 sGnRH and cGnRH-II were much lower (~100-fold) than those of sbGnRH (Amano et al.,
488 2004). In turbot, pituitary sbGnRH content was up to 600-fold higher than sGnRH at the
489 spawning season, whereas pituitary cGnRH-II content was not detectable (Andersson, et al.,
490 2001). In the Japanese flounder, sbGnRH and sGnRH were the most abundant in the pituitary,
491 whereas cGnRH-II was almost undetectable (Pham et al., 2006). It is possible that the relative
492 abundance between GnRH forms in a given species varies throughout the reproductive cycle. In
493 gilthead sea bream, where only the sbGnRH and cGnRH-II forms are found in the pituitary, both
494 GnRHs showed similar levels at early maturation, but as gonadal development advanced
495 sbGnRH levels increased significantly while cGnRH-II remained unchanged (Holland et al.,
496 1998). Contrarily, in Japanese flounder sbGnRH pituitary levels were high at previtellogenesis
497 and low at early spawning (Pham et al., 2006). In male European sea bass, sbGnRH was highest
498 at the beginning of the spawning season, but decreased significantly through the next few months
499 (Rodríguez et al., 2000). The present data should be confirmed in future studies, by analyzing
500 GnRH gene expression and peptide levels throughout the reproductive cycle of S. sole.
501 The pituitary GnRH levels were similar between wild and cultured broodstocks, both in
502 females and males. Nevertheless, wild females showed sbGnRH levels 2-fold higher than those
503 of cultured. The sbGnRH is believed to be the most relevant form in the regulation of GTH
504 secretion in fish species that express three GnRH forms (Lethimonier et al., 2004), including
505 pleuronectiformes (Andersson et al., 2001; Amano et al., 2004; Pham et al., 2006). Previous
506 studies have suggested a negative effect of captivity on the sbGnRH system, maybe related to a
507 failure in gonadal maturation. Cultured stripped bass, which fail to undergo maturation after
508 vitellogenesis, displayed low sbGnRH gene expression and pituitary content (Zohar and

Mylonas, 2001). Similar circumstances were also suggested for the pleuronectiforme Barfin flounder that do not spawn spontaneously under rearing conditions (Amano et al., 2002). Therefore, it would be possible that the lower, albeit not significant, sbGnRH levels found in cultured female S. sole breeders could have a detrimental effect on GTH synthesis and secretion and be related to the inhibition of further reproductive events.

Successful reproduction depends on the normal ontogenesis of the BPG axis, a complex process that starts early in life. Although this process is predetermined by genetic factors, young fish are vulnerable to environmental (physical and chemical) dynamics that can affect the endogenous endocrine axis, disturbing or even overriding the putative developmental pathway (Okuzawa, 2002; Strüssman and Nakamura, 2002). In our study, it should be stated that the only life episode in which the wild and cultured generations did not share similar living (rearing) conditions was from hatching to the juvenile stage, when wild soles were caught. This fact seemed to determine drastically the further reproductive effectiveness of the breeders. Further studies focused on the ontogeny of the BPG axis in S. sole under natural and artificial conditions should be addressed, in order to determine any critical sensitive period which might compromise further reproductive success of adult S sole.

In conclusion, this study described the cloning of S. sole gonadotropin subunits and the development of specific qPCRs for their pituitary mRNA quantification. For the first time, it was determined the coexistence of three GnRH forms (sbGnRH, sGnRH, and cGnRH-II) in the pituitary of S. sole, displaying a relative abundance of cGnRH-II > sbGnRH > sGnRH. Our data suggest that both FSH and LH might be involved in the regulation of gonadal maturation of male and female S. sole during the spawning period. Wild S. sole breeders, both males and females, showed biochemical, histological and gene expression signs of a more active reproductive process compared to cultured breeders; although these differences might not probably explain the reproductive dysfunctions found in cultured S. sole broodstocks.

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711

712 **Figure legends**

713 Fig. 1. Nucleotide and deduced amino acid sequences of S. sole FSHβ (A), LHβ (B) and
714 GPα (C) gonadotropin subunits. The nucleotide number is shown on both sides. Amino acids are
715 given as single capital letters bellow the cDNA sequences, with the signal peptide sequence
716 underlined. The nucleotide polyadenilation signals are underlined and the stop codon indicated by
717 an asterisk. Putative N-glycosilation sites are marked by a grey box. The nucleotide sequences
718 have been previously presented as a symposium short communication (Guzmán et al., 2007) and
719 deposited in the GenBank DNA databases under accession numbers, **EF617341**, **EF617342** and
720 **EF617342**, for LHβ, FSHβ and GPα, respectively.

Fig. 2. Localization of gonadotropin subunit mRNA expressing cells in the pituitary S. sole males (A, B, C, D, G, H, I) and females (E, F, J, K), by in situ hybridization (ISH), using specific S. sole FSHβ, LHβ and GPα probes. The GPα gene transcripts were localized in small rounded cell populations, in the border and medial region of the PPD (A and B), as well as in the border of the PI, as rounded and polygonal shape cell populations (C, D and E). The expression of FHSβ and LHβ was restricted to the border of both the PPD and the PI (G, I and J). Note the absence of transcript expression in the RPD (A and B). Sense riboprobes showed negative in situ signals (F, H and K). Abbreviations: PPD, proximal pars distalis; RPD, rostral pars distalis; PI, pars intermedia; NH, neurohypophysis. Scale bars: 100 μm.

Fig. 3. Pituitary mRNA levels of FSHβ (A), LHβ (B) and GPα (C) subunits in wild versus cultured female (left) and male (right) S. sole broodstock at the spawning time, analyzed by qPCR. Levels were normalized to S. sole 18s and represented as mean ± SEM (n=4). Different capital letters indicate differences ($p<0.05$) among subunits in wild S. soles; different small letters indicate differences ($p<0.05$) among subunits in cultured S. soles. For each graph, * indicates significant differences ($p<0.05$) between generations (wild versus cultured). For each subunit, # indicates significant differences between sexes in the same generation broodstock (wild or cultured).

Fig. 4. Pituitary content of sbGnRH (A), sGnRH (B) and cGnRH-II (C) forms in wild versus cultured female (left) and male (right) S. sole broodstock at the spawning time, analyzed by ELISA. Data are represented as mean ± SEM (n=4). ND = non detectable levels. Different capital letters indicate differences ($p<0.05$) among GnRH forms in wild S. soles; different small letters indicate differences ($p<0.05$) among GnRH forms in cultured S. soles. For each graph, * indicates significant difference ($p<0.05$). For each GnRH form, # indicates significant differences between sexes in the same generation broodstock (wild or cultured).

Fig. 5. Photomicrographs of cross sections of S. sole ovaries showing oocytes at different developmental stages, from wild (A to F) and cultured (G and H) females. In wild females, it is shown oocytes at different stages of vitellogenesis (A and B), early maturation (C) and atresia (D and F), as well as postovulatory follicles (E). Early degeneration (D) and advanced stages (F) of atresia, in which the follicular layer appears hypertrophied and the zona radiata highly fragmented, is shown. Oocytes of cultured females displayed a reduced proportion of maturating oocytes in favour of those in vitellogenesis (G and H). Abbreviations: N, nucleus; Nu, nucleolus; YG, yolk granules; Yc, yolk coalescence; L, lipid droplets; ZR, zona radiata; FE, follicular envelope; PoF, pots ovulatory follicles; AO, atretic oocytes. Scale bars: 100 μm.

754 Fig. 6. Histological sections of S. sole testis at the functional maturation stage, from wild (A
755 and B) and cultured (C and D) males. Details of cortex (A and C) in the periphery and medulla (B
756 and D) in the internal or central region of testis are shown. Note the increase of the lobular lumen
757 size and the accumulation of spermatozoids in the testis of wild fish (B) when comparing to those
758 of cultured (D). Abbreviations: IT, interstitial tissue; spg, spermatogonia; spc, spermatocyte; spd,
759 spermatid; spz, spermatozoa. Scale bars: 50 μm.

760 Fig. 7. Gonadosomatic index (GSI) of females (left) and males (right) from wild versus
761 cultured S. sole broodstock at the spawning time. Data are represented as mean ± SEM (n=4). For
762 each graph, * indicates significant differences ($p<0.05$).

763 Fig. 8. Plasma levels of E2, T and VTG in female (left) and 11-KT and T in male (right) S.
764 sole broodstock (wild versus cultured), at the spawning time. Data are represented as mean ±
765 SEM (n=4). For each graph, * indicates significant differences ($p<0.05$).

766

Table 1. Primers used to isolate the S. sole FSHβ, LHβ and GPα subunit cDNAs, by Smart RACE RT-PCR.

Tanscript	Primer	Direction	5' to 3' primer sequence
FSHβ	FSH-FW [a]	Forward	5' GATGVAGYTRGTYSTCATGG 3'
	ssFSHr [c]	Reverse	5' CATAGGTCCAGTCCCCGTT 3'
LHβ	LH-RV [a]	Reverse	5' GGCTGNAGRCTCTCRAAGGT 3'
	ssLHf [c]	Forward	5' CTCTGGAGAAGGACGGATGT 3'
GPα	GP-RV [b]	Reverse	5' GTGGCAGTCTGTGTGGTTCC 3'
	ssGPf [c]	Forward	5' TGCTGCTTCTCCAGAGCGTA 3'

[a] Degenerated primers previously used in the three-spined stickleback (Hellquist et al., 2004). R=G or A, Y= T or C, S=G or C, N=G, A, T or C.

[b] Primer designed based on a consensus sequence of GPα from Atlantic halibut (**AJ417770.1**), Japanese flounder (**AF268692**) and European sea bass (**AF269157.1**); sequences are available at Gen Bank.

[c] S. sole gene specific primers used for the second round RACE-PCR.

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768

768

Table 2. Gene-specific primers, amplicon size (bp) and PCR efficiency for each transcript in the quantitative real time PCR assays.

Transcript	Primer	Nucleotide sequence	Size (bp)	Efficiency (%)
FSHβ	ssFSHf_q	5' GGACCCAAACTACATCCATGAAC 3'	60	102.3 ± 2.1
	ssFSHr_q	5' CAGTCCCCGTTACAGATCACCTGTCT 3'		
LHβ	ssLHf_q	5' CGGTGGAGACGACCATCTG 3'	61	102.5 ± 2.0
	ssLHr_q	5' GGTATCTTGATGACGGGATCCTT 3'		
GPα	ssGPf_q	5' ACGGGCTGTGAGAAATGCA 3'	56	96.8 ± 3.5
	ssGPr_q	5' GGATGCTCCCTGGAGAACAA 3'		
18s[a]	18Sf_q	5' GGCGGCGACGTCTCATT 3'	61	87.5 ± 1.3
	18Sr_q	5' GTAGGCACAGAAAGTACCATCGAA 3'		

[a] Gene-specific primer designed from the S. sole 18s complete gene sequence, available at Gene Bank (**EF126042.1**).

769

(A) ssFSHβ

```
  1                        GGAACGCTGGTACAGATGTTCAGAGAGTTAAAGAAGGAGA   40
 41  AAGATTGGCACCTGAGGTTCAACAGAGCTTCAAAGGCGTCTGTACTGCAACCAGCAGAGG  100
101  ATGCAGCTGGTTGTCATGGCAGCAGTGCTGGCAATAGCGGGGGCGGGGCAGAGCTGCAGC  160
       M  Q  L  V  V  M  A  A  V  L  A  I  A  G  A  G  Q  S  C  S
161  TCCAGATGTCGTCCAGCCAACGTCAGCATCCCCGTGCAGAGCTGTGGCAACACCGAGTAT  220
       S  R  C  R  P  A  N  V  S  I  P  V  Q  S  C  G  N  T  E  Y
221  ATCTACACCACTATGTGTGCAGGACAGTGCTACCACGAGGACCCAAACTACATCCATGAA  280
       I  Y  T  T  M  C  A  G  Q  C  Y  H  E  D  P  N  Y  I  H  E
281  CTTGGCATGGATAGACAGGTGATCTGTAACGGGGACTGGACCTATGAAGTGAAACGCATT  340
       L  G  M  D  R  Q  V  I  C  N  G  D  W  T  Y  E  V  K  R  I
341  AATGGATGTCCACAGGCAGTGACCTACCCTGTGGCCACAAACTGCCACTGTACTTCATGT  400
       N  G  C  P  Q  A  V  T  Y  P  V  A  T  N  C  H  C  T  S  C
401  AATCCAGACAACACTCACTGTGGCCGCTTTCCTGGAGAGATTGCCAGCTGTCTGTCCTTT  460
       N  P  D  N  T  H  C  G  R  F  P  G  E  I  A  S  C  L  S  F
461  TAA                                                         463
       *
464  AAAGAAAACAAACAAAAAACAGTGTACATTCTTATATTTCCCTGTTTCCCTTGACATGGG  523
524  ACTGAAAAATAAACGCTTCGCTGCT(-polyA)                           548
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(B) ssLHβ

```
  1                                                GGGACAAAGACACAGAGGAAC   21
 22  ATGGCCGCCGTGCAGATCAGGAGGTTGACCTTTCACCTGACGCTCACCGTCCTCCTAGGA   81
       M  A  A  V  Q  I  R  R  L  T  F  H  L  T  L  T  V  L  L  G
 82  TCCTCGACGTCCGATTGGCCGCCGGCTCCTGCAGTGTCCTCTCAGCTGTCTCCGTGTCAG  141
       S  S  T  S  D  W  P  P  A  P  A  V  S  S  Q  L  S  P  C  Q
142  CTTGTCAACCAAACGGTGTCTCTGGAGAAGGACGGATGTCACACGTGTCACCCGGTGGAG  201
       L  V  N  Q  T  V  S  L  E  K  D  G  C  H  T  C  H  P  V  E
202  ACGACCATCTGCAGCGGCCACTGCTACACCAAGGATCCCGTCATCAAGATACCATTCCTC  261
       T  T  I  C  S  G  H  C  Y  T  K  D  P  V  I  K  I  P  F  L
262  AACATGTACCAGCACGTGTGCACGTACCAGGACGTGCACTACAAGACCTTTCACCTGCCG  321
       N  M  Y  Q  H  V  C  T  Y  Q  D  V  H  Y  K  T  F  H  L  P
322  GACTGTGGTCCCGGCGTGGACCCGAGCGTCACGTACCCGGTGGCTGTGAGCTGCAGCTGC  381
       D  C  G  P  G  V  D  P  S  V  T  Y  P  V  A  V  S  C  S  C
382  AGCAGGTGTGCGATGGACATGTCCGACTGCACGTATGAGAGCCTGCAGCCCGACATCTGC  441
       S  R  C  A  M  D  M  S  D  C  T  Y  E  S  L  Q  P  D  I  C
442  ATGAACGACATACTTTTCTACTACTGA                                 468
       M  N  D  I  L  F  Y  Y  *
469  CGTCTGCAAACTATAATAAAAATAATATAGTAACTACATAATAATGATGACAGAGTTAAAC  529
530  AATGTGGCTCAACAGCCACTTATATACAAATAAAAACCAGTG(-polyA)              571
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(C) ssGpα

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  1                                GCTCAGTATCAGGAGCCCTGCACAGACGCACG   32
 33  ATGAAGGGGACGCTTTCTCTCAACATGGCAGCCGCTACAACAACAGTGGGTTCGGTAAAA   92
       M  K  G  T  L  S  L  N  M  A  A  A  T  T  T  V  G  S  V  K
 93  TCAGTGGGGCTGTCTCTTCTTCTATTGTCTTTTTTTCTTTACATAGCTGATTCTTACCCC  152
       S  V  G  L  S  L  L  L  L  S  F  F  L  Y  I  A  D  S  Y  P
153  AACATTGAACTACCAGACACGGGCTGTGAGAAATGCACCCTGAGGAAGAGCCATTTGTTC  212
       N  I  E  L  P  D  T  G  C  E  K  C  T  L  R  K  S  H  L  F
213  TCCAGGGAGCATCCGGTCTACCAGTGCAAGGGCTGCTGCTTCTCCAGAGCGTACCCGACG  272
       S  R  E  H  P  V  Y  Q  C  K  G  C  C  F  S  R  A  Y  P  T
273  CCGCTCAAGGCGATGCAGACGATGCCGATCCCCAAGAACATCACGTCAGAGGCGACGTGC  332
       P  L  K  A  M  Q  T  M  P  I  P  K  N  I  T  S  E  A  T  C
333  TGTGTCGCGAAGCACAGCTACGAGACGGAGGTGGCCGGCATCCGGGTGAAGAACCACACA  392
       C  V  A  K  H  S  Y  E  T  E  V  A  G  I  R  V  K  N  H  T
393  GACTGTCACTGCAGCACCTGTTTTTTCCACAAGATTTGA                     431
       D  C  H  C  S  T  C  F  F  H  K  I  *
433  CCAACGAGAGAACCGGAGACCACTCGGCAGCGCTCGGCCCCACGACGACCTCTGTGTGTT  492
493  TTAATGTGCAAAAGCTCTTCTTCTTCTTCTCTCTTTCAGTATATATACATATATGTATAT  552
553  ATATGTATATATACATATATATATGTATATGTATGCATGTTCTCAAATTGCCAGATGATA  612
613  TTTTTGTAGCAGTTCAGTGCCTGTGGTGTGTAATTAGACCATGATTTTTTTTTGTTCCAT  672
673  TGATAGATGACTAACAGTATCATAGAAAGCAAATAAACATATGAACAC(-polyA)       719
```

Fig. 1

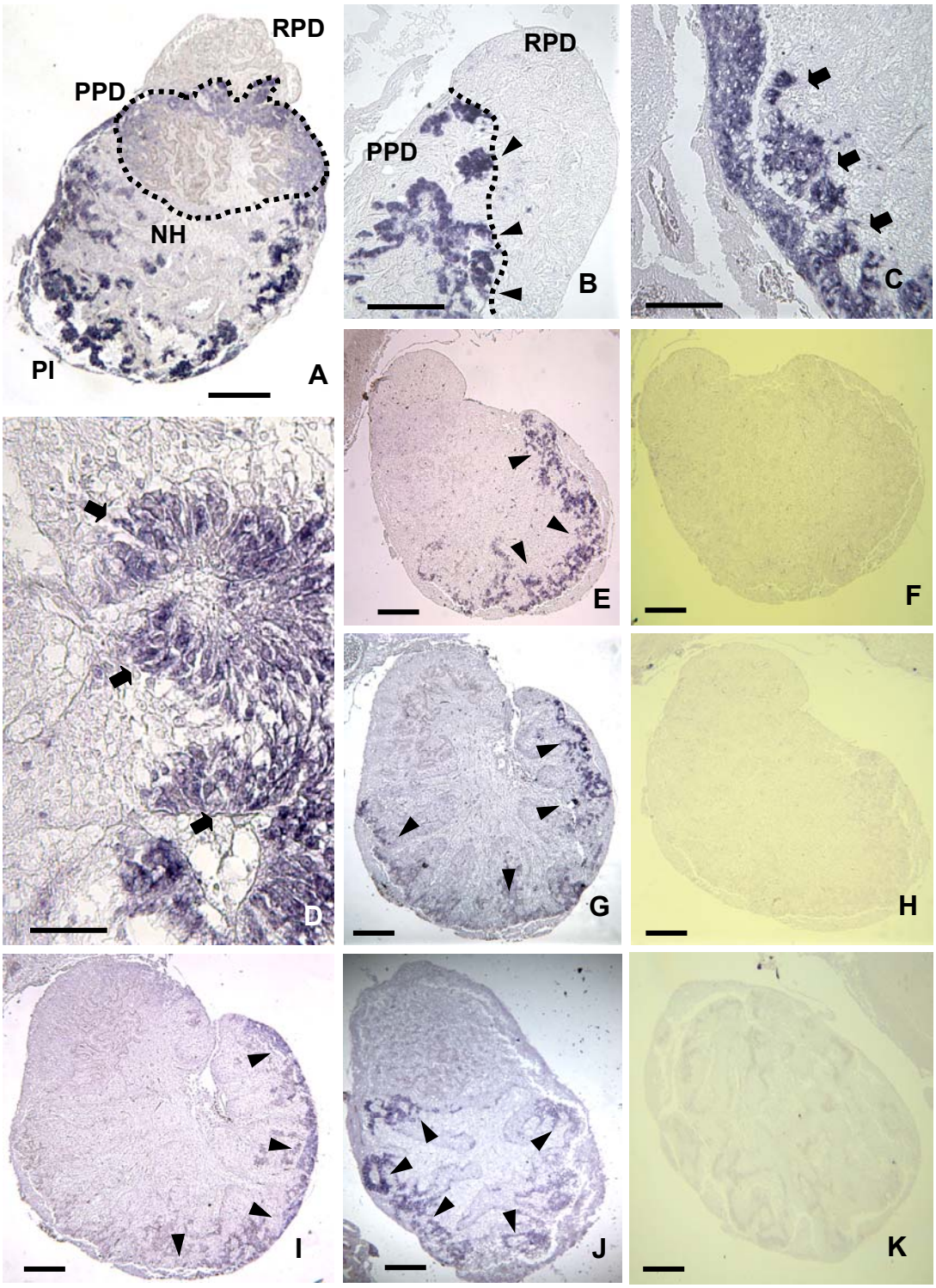
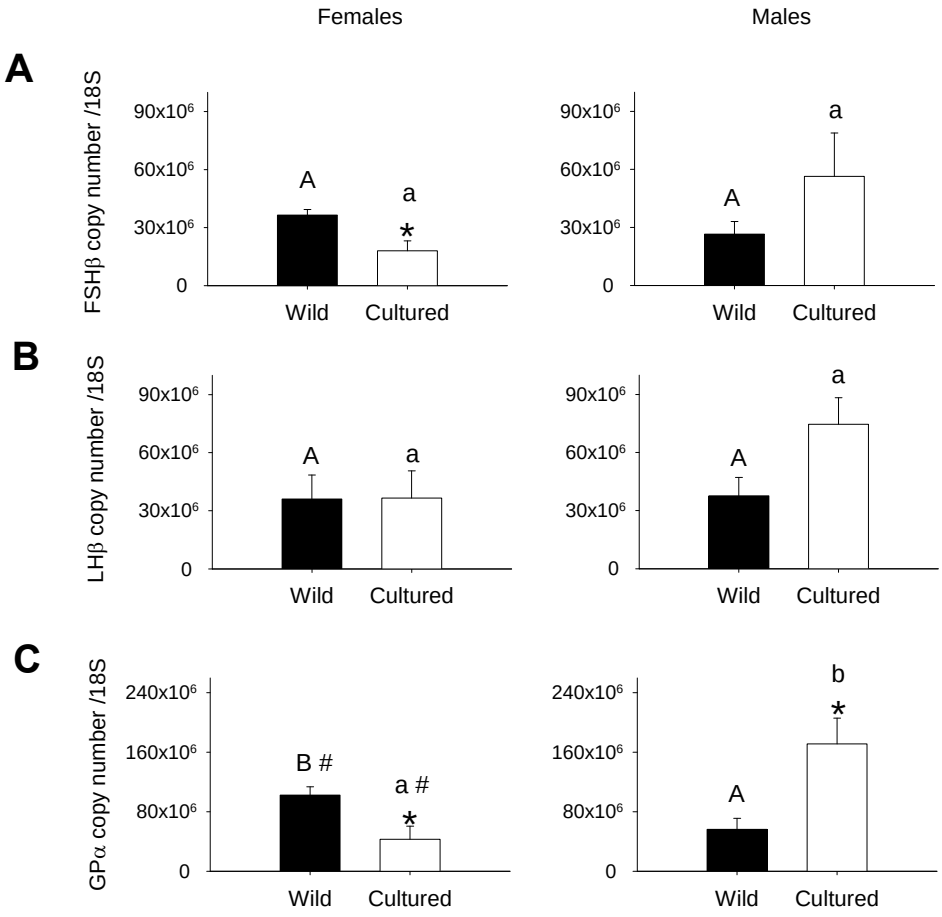


Fig. 2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a
*
Wild
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a
B
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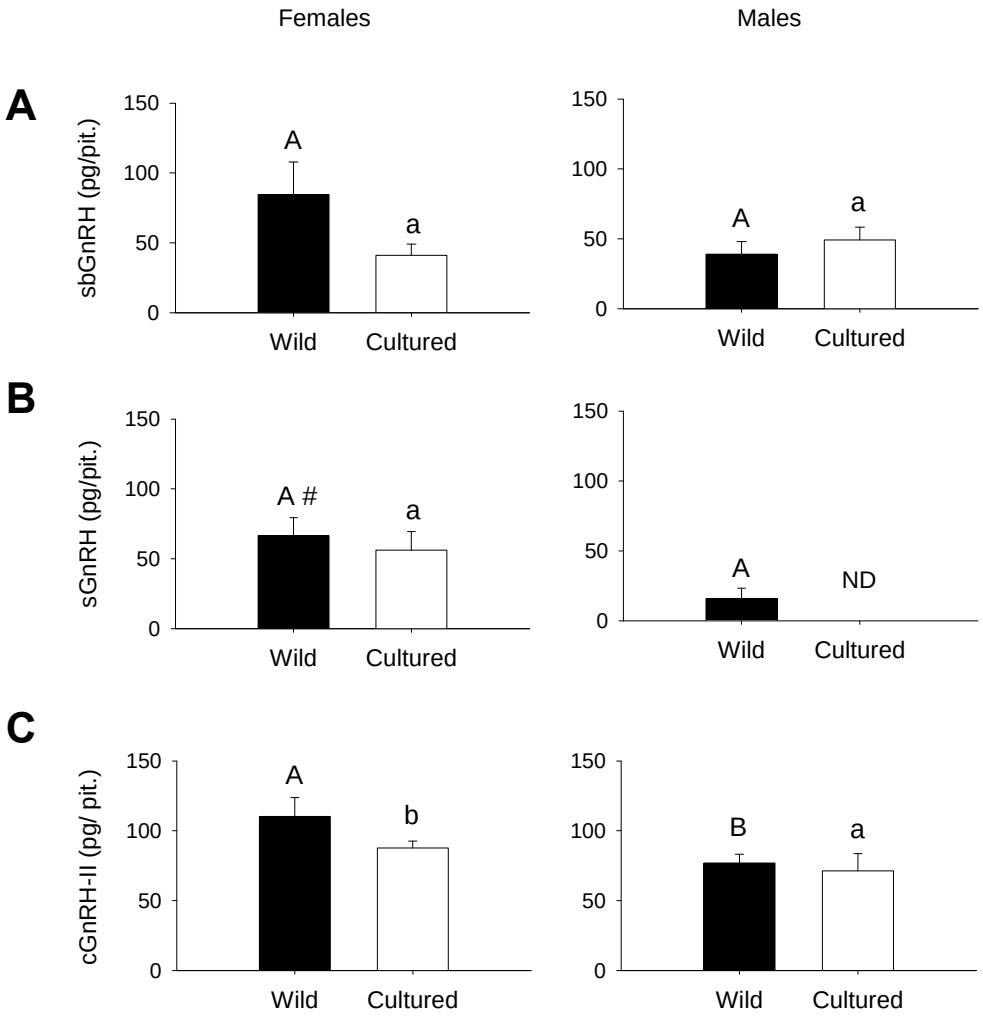
B #
a #
b

Fig. 3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Cultured
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Cultured
B
sGnRH (pg/pit.)
150
100
50
0
A #
a
A
ND
Wild
Cultured
Wild
Cultured
C
cGnRH-II (pg/ pit.)
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b
B
a
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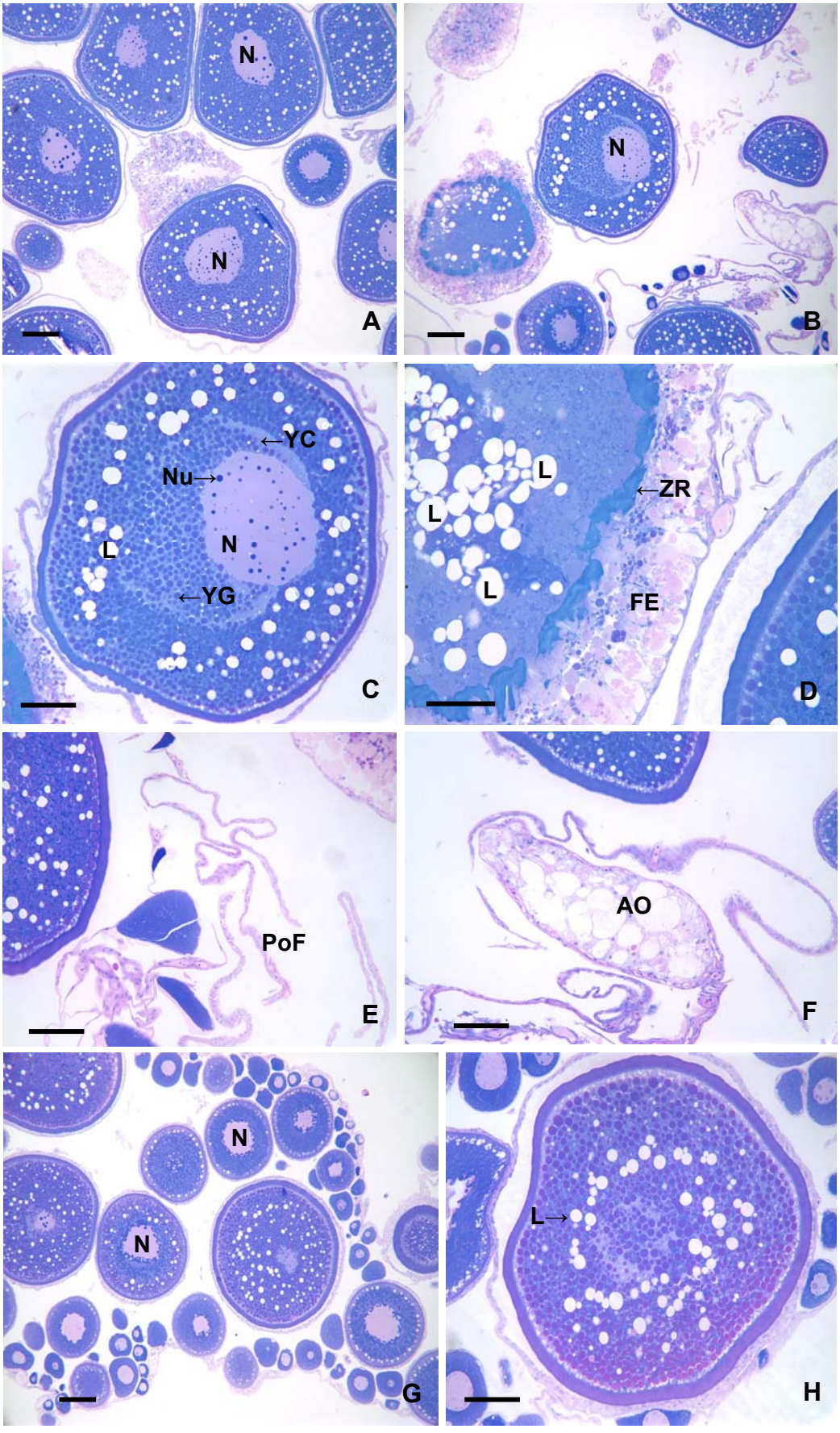
Wild
Cultured

Fig. 4



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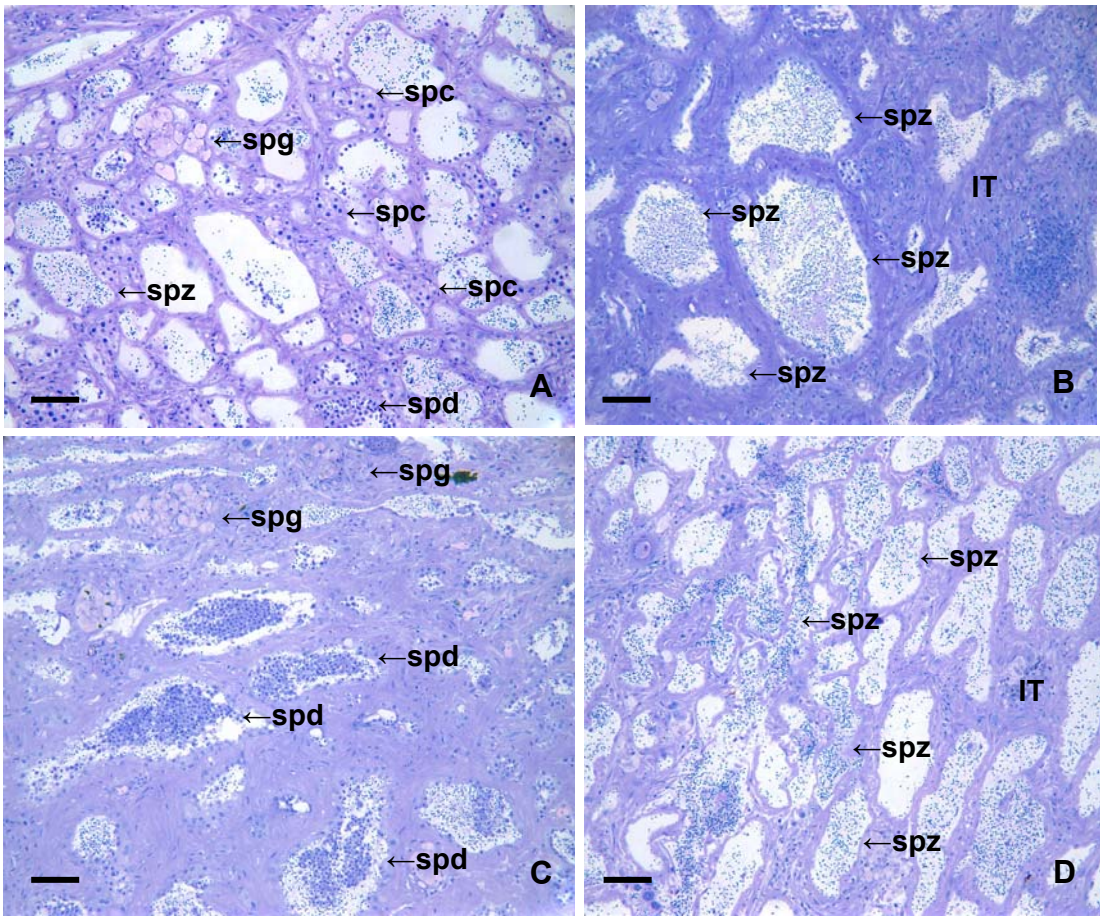
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D

Fig. 6

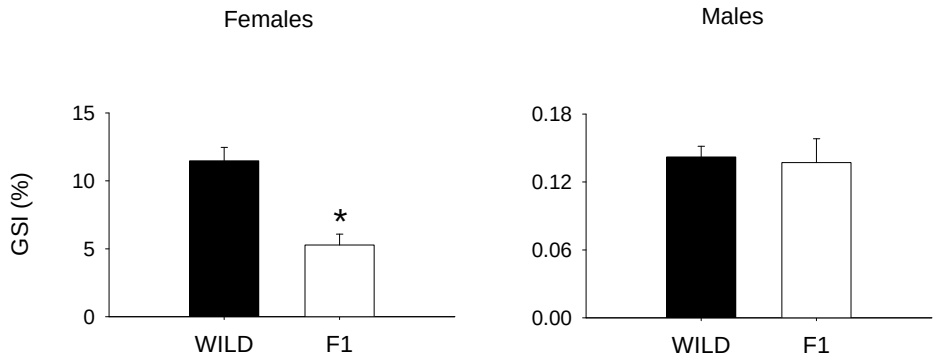
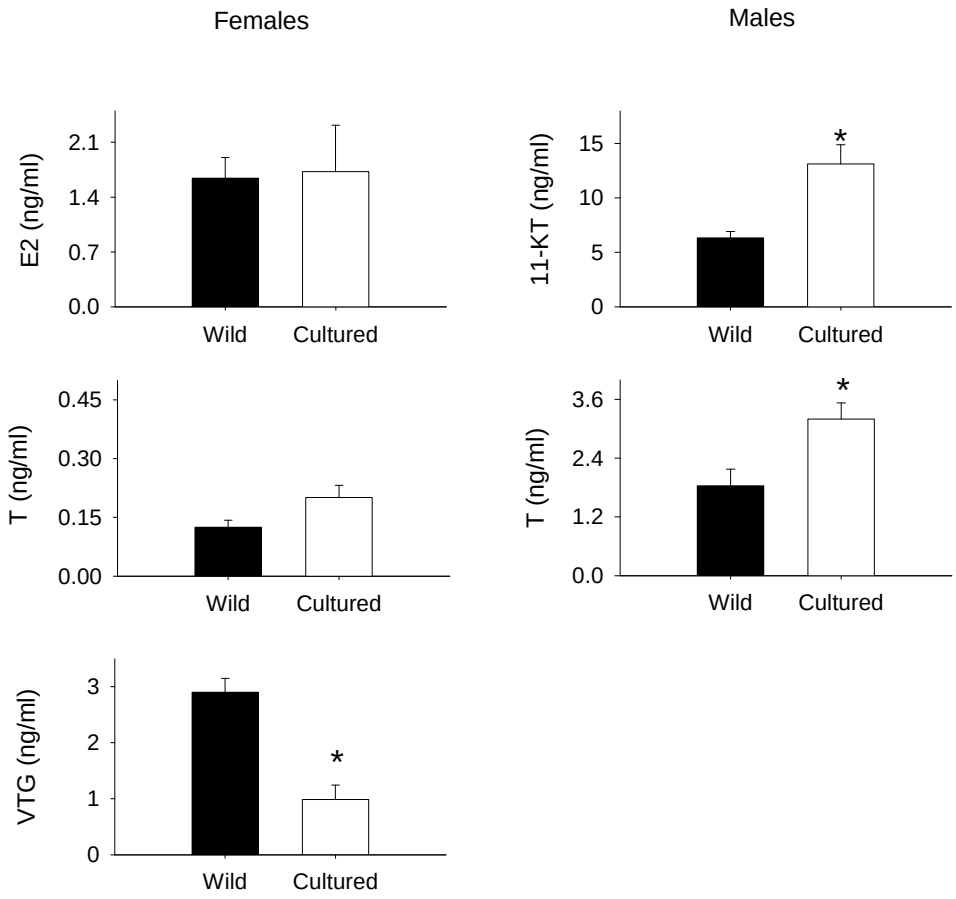


Fig. 7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Wild
Cultured
11-KT (ng/ml)
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10
5
0
*
T (ng/ml)
0.45
0.30
0.15
0.00
3.6
2.4
1.2
VTG (ng/ml)
3
2
1

Fig. 8